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I (name and address of translator) Ing. Estera **RUBINOVA**

Lastovičia 15, 940 72 **NOVE ZAMKY** (Slovak Republic)

am the translator of the document(s) attached and I state that the following is a true translation to the best of my knowledge and belief.

E. Rubinova

Ing. Estera Rubínová - GAMMA
Konzultácie a preklady
Lastovičia 15
940 72 Nové Zámky
IČO: 41 762 576 DIČ: 1075471342

FUNCTIONALIZING A PEPTIDE WITH AN α-HYDRAZINOACETIC GROUP

The present invention is related to a homogenous phase coupling process between a peptide and at least one compound bearing a carboxylic acid or alcohol function, such as a lipid, a sugar, an alcohol or a fluorescence marker, as well as to modified peptides which are mainly constituted by a peptide linked by a hydrazide bond to at least one compound as defined above.

The present invention is also related to the use of N,N'-tri(Boc)hydrazinoacetic acid or of N,N'-di(Boc)hydrazinoacetic acid for functionalizing a peptide with an α-hydrazinoacetic group.

The problem to enter into living cells of different substances with pharmacological properties is of major therapeutic importance. Synthetic peptides and oligonucleotides encounter difficulties to pass through the cellular membrane. An interesting approach aimed at improving their ability to penetrate a cell is that of modifying thereof with a lipophilic part. It has thus been shown that a peptide modified by a simple aliphatic chain is capable of penetrating the cell by passive transfer through the membrane, and of interacting with its intracytoplasmic target. Therefore, lipopeptides are molecules of interest for the purpose of vectorizing a functional moiety within the cell.

Lipopeptides synthesis can be carried out, for example, by solid phase coupling of a fatty acid to a peptide. Upon completion of the synthesis, steps of cleavage of the peptide/solid support bond and of deprotection of the peptide side chains using a strong acid have to be carried out. This treatment considerably restricts the choice of the lipophilic part ; it prevents, in particular, the use of unsaturated fatty acids. Moreover, the purification of

lipopeptides by reverse phase high-performance liquid chromatography is difficult and leads to low yields, given the numerous impurities that are present at the end of synthesis.

Homogenous phase coupling of a protein to a palmitoyl-coenzyme A group, the latter being introduced into the thiol group of a cysteine, has also been suggested. Such a coupling leads to the formation of a thioester link, which has the drawback of being unstable. On the other hand, this strategy is limited to the modification of certain proteins by palmitoyl-coenzyme A and cannot be generalized for the synthesis of lipopeptides.

Present lipopeptides synthesis strategies also involve the use of chemical ligation reactions. Chemical ligation enables to link, in homogenous phase and under extremely mild conditions, two previously purified and completely deprotected peptide structures.

Thus, it has been suggested to link a fatty acid to a peptide with a disulfide bond in an aqueous buffer. However, the disulfide bond creates many problems ; such a bond is, in fact, unstable and liable to be degraded in the presence of thiols, whence the need to avoid contaminating the solvents used to solubilize the products with thiols, as well as the impossibility of introducing a cysteine into the peptide sequence to be vectorized. Moreover, the use of the thiol chemistry requires working in an inert atmosphere in order to prevent oxidization of the thiols.

W. ZENG *et al.* (*J. Pept. Sc.*, 1996, 2, 66-72) have also suggested homogenous phase coupling of a completely deprotected and previously purified peptide to a polyfunctional lipidic structure linked to a peptide, this being effected via an oxime link. The lipophilic part is introduced into a peptidic sequence in solid phase, such a method having the aforementioned

drawbacks, namely the limitation of the choice of the lipophilic part, and difficulties associated with purification of the lipidic structure.

Similarly, O. MELNYK *et al.* (*J. Peptide Res.*, 1998, 52, 180-184) have described homogenous phase ligation and via a hydrazone bond of a lipophilic aldehyde of peptidic nature and of another peptide modified at the lysine side chain level with a hydrazino group. The hydrazone bond is produced in homogenous phase, but the lipophilic aldehyde is solid phase synthesized, and the limitations are the same as those previously described. In addition, the hydrazone bond is sensitive to acidic conditions.

Chemical ligation appears to be an excellent method for lipopetide synthesis permitting an improvement in yields obtained for these compounds. However, we have seen that there are no ligation methods, at the present time, not using thiol chemistry and enabling a direct coupling of a lipophilic compound, not bound to a carrier structure, to a completely deprotected peptide.

The Inventors thus assigned themselves the task of providing a new strategy for the synthesis of lipopeptides and, in general, of peptides modified by different compounds of lipidic or other nature, by homogenous phase chemical ligation.

This new synthesis strategy should, in particular, meet the following criteria;

- the coupling of the above-mentioned compound, for example a lipid, to the peptide, takes place in homogenous phase,
- the coupling is carried out with a completely deprotected peptide, the reaction being chemoselective,
- the reaction conditions of coupling enable to use directly fatty acids and commercial cholesterol derivatives,

- the reaction conditions of coupling enable, in particular, the introduction, into the peptide, of carboxylic acids and sensitive alcohols such as, for example, mono- and polyunsaturated complex fatty acids and cholesterol derivatives,
- the link formed in the course of coupling is very stable over a large range of pH values.

The Inventors also assigned themselves the task of providing modified peptides, capable of being obtained by chemical coupling, wherein said peptides are linked to different compounds, in particular lipids, by a very stable linkage not having the drawbacks of the disulfide bonds of the prior art.

These objects are obtained by creating a hydrazide bond between the peptide and the compound linked thereto in a convergent homogenous phase synthesis.

The present invention is related to a coupling process of a peptide and at least of one compound A, of non-peptidic nature, bearing a function selected from the group formed by carboxylic acid functions and alcohol functions, characterized in that said coupling includes a step of producing, in homogenous phase, a hydrazide bond between said peptide and said compound A.

Within the meaning of the present invention, "peptide" refers to any coupling of several amino acids, whatever their nature and number; thus, "peptide" refers to both oligopeptides (dipeptides or tripeptides) and polypeptides or proteins. Equally, "hydrazide bond" refers to a covalent bond including the moiety -CO-NH-NH-.

In a particularly advantageous way, the process according to the invention, which is carried out in homogenous phase, enables to avoid a step of cleavage of the modified peptide obtained from the support, which cleavage, as we have already seen, considerably

restricts the choice of the compound linked to said peptide. Furthermore, the hydrazide bond produced between the peptide and the compound, or compounds, A is very stable, and this over a very wide range of pH values, and *in vivo*.

According to an advantageous form of embodiment of the coupling process according to the present invention, the latter includes, for the purpose of producing said hydrazide bond, the following steps :

a) activation of the function borne by said compound A into a corresponding reactive function, selected from the group formed by ester functions and carbonate functions respectively, when the compound A bears a carboxylic acid function and an alcohol function respectively ; and

b) reaction, in homogenous phase and at a pH of less than 6, between said activated compound A obtained in a) and a completely deprotected peptide, bearing at least one hydrazine or hydrazine derivative group, either at its N-terminal end or at the end of a lysine or an ornithine side chain possibly present at any point in the peptide sequence.

Within the meaning of the present invention, a "hydrazine group" or "a hydrazine-derived group" refers to the moiety $-NH-NH_2$.

A hydrazine group can be introduced either at the N-terminal end of the peptide or at the end of a lysine or an ornithine side chain possibly present at any point in the peptide sequence, by any means known to a person skilled in the art, for example according to an N-amination protocol as described by C. KLINGUER *et al.*, in Tetrahedron Letters, 1996, 37, 40, 7259-7262.

In a particularly advantageous way, the reaction between said activated compound A and said completely deprotected peptide, functionalized as described above, enables to avoid any deprotecting step of the peptide

side chain with a strong acid, considerably limiting, as it has been previously shown, the choice of the compound coupled to said peptide. Thus, the reaction of the said compound A and the said completely deprotected peptide, functionalized as described herebove, enables to obtain directly the modified peptide, i.e., the peptide linked to compound A.

The process according to the invention enables to carry out a chemoselective reaction of the functional group (hydrazine group or hydrazine derivative group) introduced into the peptide and the activated compound or compounds A ; the reaction takes place, in fact, at a pH lower than 6, a pH such that the amino functions of the lysine (ε-NH_2 function) or the ornithine (δ-NH_2 function) side chains or the N-terminal α-NH_2 function possibly present in the peptide sequence, are protonated, hence weakly reactive. Thus, the pH control enables to preferentially acetylate the hydrazine or hydrazine derivative group introduced into the peptide, without reacting the other functional groups of the constitutive amino acid side chains of the peptide.

The coupling reaction, carried out in course of the process according to the present invention (step b), takes place under very mild operating conditions and, in a particularly advantageous way, does not require working in inert conditions, as is the case with some processes of the prior art, in particular those consisting of coupling a peptide to a fatty acid with a disulfide bond.

According to an advantageous form of embodiment of the coupling process according to the invention, said process further includes a step c) of purification of the modified peptide obtained in step b).

Such a purification is conventionally carried out by high-performance liquid chromatography. By comparison with the purification of a modified peptide obtained by a coupling process carried out in solid

phase, as previously described, the purification of the modified peptide obtained by the coupling process according to the present invention leads to far better yields, the modified peptide obtained in step b) having a higher purity than a modified peptide obtained in solid phase.

According to another advantageous form of embodiment of the coupling process according to the present invention, after step a) of activation of the function borne by compound A, the corresponding reactive function borne by compound A is selected from the group consisting of succinimidyl, sulfosuccinimidyl and aryl esters and carbonates.

Para-nitrophenyl esters and carbonates can be cited as examples of aryl esters and carbonates.

According to another advantageous form of embodiment of the coupling process according to the invention, said hydrazine-derived group borne by the peptide is an α-hydrazinoacetic group.

According to a preferred arrangement of this form of embodiment, prior to step b) of the process according to the invention, said peptide is functionalized by an α-hydrazinoacetic group, either at its N-terminal end or at the end of a lysine or of an ornithine side chain possibly present at any point in the peptide sequence, using N,N'-tri-(Boc)-hydrazinoacetic acid or N,N'-di(Boc)hydrazinoacetic acid.

According to a preferred form of this arrangement, the functionalization of said peptide by an α-hydrazinoacetic group, by means of N,N'-tri(Boc)hydrazinoacetic acid or N,N'-di(Boc)hydrazinoacetic acid, is followed by a purification step of said peptide functionalized by high-performance liquid chromatography, using an eluent consisting of a water/alcohol mixture, preferably a water/isopropanol mixture, including trifluoroacetic

acid. Such an eluent advantageously enables to avoid any degradation of the α-hydrazinoacetic group borne by the peptide.

According to another advantageous form of embodiment of the coupling process according to the invention, said compound A is selected from the group consisting of lipids, sugars, alcohols and fluorescence markers.

As example of a usable fluorescence marker, mention can be made, non-limitatively, of fluorescein or rhodamine.

According to a preferred arrangement of this form of embodiment, said lipids are selected from the group consisting of saturated fatty acids, unsaturated fatty acids and sterols. The process according to the invention advantageously enables, in fact, to link complex (mono- and polyunsaturated) fatty acids and, generally speaking, any sensitive carboxylic acid, to a peptide. Preferably the above-mentioned lipids are selected from the group consisting of palmitic acid, stearic acid, cis-9,10-epoxystearic acid, oleic acid, linoleic acid and cholesterol.

An object of the present invention is also a modified peptide mainly consisting of a peptide linked by a hydrazide bond to at least one compound A bearing, prior to linking thereof to said peptide, a function selected in the group consisting of carboxylic acid functions and alcohol functions.

The present invention is also related to a modified peptide essentially consisting of a peptide linked with a hydrazide bond to at least one compound selected from the group consisting of lipids, sugars, alcohols and fluorescence markers.

According to a preferred arrangement of this embodiment, the modified peptide according to the present invention is an oligopeptide mainly consisting of a peptide linked with a hydrazide bond to at least

one lipid selected from the group consisting of saturated fatty acids, unsaturated fatty acids and sterols.

Preferably, said oligopeptide according to the invention consists mainly of a peptide linked with a hydrazide bond to at least one lipid selected in the group consisting of palmitic acid, steraic acid, cis-9,10-epoxystearic acid, oleic acid, linoleic acid and cholesterol.

The stability of a hydrazide bond makes particularly interesting the peptides modified according to the invention since the hydrazide bond is stable both *in vivo* and over a very wide range of pH values. Furthermore, the hydrazide bond is stable under catalytic hydrogenation conditions which enable, for example, in the case of peptides modified by unsaturated fatty acids, the synthesis of tritium-labeled lipopeptides in the fatty chain, useful for an intracellular radioactive monitoring of said lipopeptides and for better understanding the mechanism of action thereof.

The present invention also relates to a synthetic vaccine and a diagnosis reagent including at least a peptide modified according to the present invention, as described hereabove.

The present invention is also related to the use of the coupling process according to the invention, as described above, for preparing a medicament including an active ingredient of a vectorized peptidic type, useful for cellular targeting.

The present invention further relates to the use of N,N'-tri(Boc)hydrazinoacetic acid or of N,N'-di(Boc)hydrazinoacetic acid for functionalizing a peptide with an α-hydrazinoacetic group, either at the N-terminal end of said peptide, or at the end of a lysine or an ornithine side chain, possibly present at any point of the peptide sequence.

It is clearly understood, however, that an α-hydrazinoacetic group can be introduced into said peptide either at the N-terminal end of said peptide or at the end of a lysine or an ornithine side chain possibly present at any point in the peptide sequence, using any process known to a person skilled in the art ; for example, functionalization of a peptide with an α-hydrazinoacetic group can be carried out via a solid phase N-amination reaction, as described by C. KLINGUER *et al.*, in Tetrahedron Letters, 1996, 37, 40, 7259-7262, by means of the commercial reagent N-Boc-3-(4-cyanophenyl)oxaziridine (BCPO). This is the case, for example, of an N-amination reaction carried out on a glycine residue in N-terminal position of a peptide or of a lysine or an ornithine side chain present at any point in the peptide sequence.

However, given the high cost of the BCPO and the very long periods of time required by such a reaction, this method of synthesis is only suitable for functionalizing products with high added value, synthesized in small amounts. In a particularly advantageous way, the use of N,N'-tri(Boc)-hydrazinoacetic acid or of N,N'-di(Boc)-hydrazinoacetic acid according to the present invention is more simple and far less expensive for functionalizing a peptide with an α-hydrazinoacetic group. This functionalization is carried out in solid phase, the functionalized peptide is then separated from the solid support and deprotected by methods known to a person skilled in the art; a purification step by high-performance liquid chromatography can be carried out using the already described water/alcohol eluent, advantageously enabling to avoid any degradation of the α-hydrazinoacetic group borne by the peptide.

Besides the foregoing arrangements, the invention also includes other arrangements which will emerge from the following description, with reference to examples

of embodiments of the process of the present invention and of syntheses of peptides modified according to the present invention, as well as to the annexed drawings, in which:

Fig. 1 illustrates the synthesis of N,N'-tri(Boc)hydrazinoacetic acid and N,N'-di(Boc)-hydrazinoacetic acid 4';

Fig. 2 illustrates the synthesis of a hydrazinopeptide 6 from a peptide 5 and N,N'-tri(Boc)hydrazinoacetic acid;

Fig. 3 illustrates the synthesis of lipopeptides 11, 12, 13, 14, 16 and 18 according to the process of the present invention, from hydrazinopeptide 6 and lipids 7, 8, 9, 10, 15 and 17, with Su being a succinimidyl group;

Fig. 4 illustrates the synthesis of lipopeptide 13 by catalytic hydrogenation of lipopeptide 12;

Fig. 5 illustrates the synthesis of lipopeptide 21 using the process according to the present invention;

Fig. 6 illustrates the synthesis of lipopeptides 23 and 24 using the process according to the present invention.

It should be understood, however, that these examples are given purely by way of illustration of the object of the invention and are not be understood as a limitation thereof.

In the following examples, the used abbreviations are:

eq.: equivalents ; Boc: *tert*-butyloxycarbonyl ; Boc_2O: di(tert-butyloxycarbonyl) ether ; CH_2Cl_2: dichloromethane ; AcOH: acetic acid ; AcOEt: ethyl acetate ; Na_2SO_4: sodium sulfate ; KH_2PO_4: potassium dihydrogenophosphate ; Na_2HPO_4: disodium phosphate ; DMF: dimethyl formamide; DMAP: 4-dimethylaminopyridine ; PEG: polyethyleneglycol ; PS: polystyrene ; $CDCl_3$: deuterated chloroform ; CD_3CO_2H:

acetic acid d_3 ; TFA: trifluoroacetic acid ; Et_2O: diethylether ; THF: tetrahydrofuran ; HBTU: N-[(1H-benzotriazol-1-yl) (dimethylamino)methylene]-N-methylmethanaminium-hexafluorophosphate N-oxide ; HOBt: N-hydroxy-benzotriazole ; ^tBu: *tert*-butyl ; DIEA: diisopropyl-ethylamine ; Pmc: 2,2,5,7,8-pentamethylchroman-6-sulfonyl ; Trt: trityl ; Fmoc: 9-fluorenylmethoxy-carbonyl ; Pbf: 2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl ; BOP: benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphoniumhexafluorophosphate ; HPLC: high-performance liquid chromatography ; RP-HPLC: reverse phase high-performance liquid chromatography ; ES-MS: electrospray mass spectrometry ; TOF: time-of-flight ; MALDI: Matrix-Assisted Laser Desorption Ionisation ; NMR: Nuclear Magnetic Resonance ; TOCSY: Total Correlation Spectroscopy ; PDMS: Plasma Desorption Mass Spectrometry ; PAL: peptide-amide linker.

EXAMPLE 1: Synthesis of N,N'-tri(Boc)hydrazinoacetic acid 4 and of N,N'-di(Boc)hydrazinoacetic acid 4' (Figure 1)

1) Synthesis of N,N'-tri(Boc)hydrazinoacetic acid 4

- *Synthesis of ethyl N-Boc hydrazinoacetate 2*

1.99 g (12.8 mmoles) of commercial ethyl hydrazinoacetate 1 and 3.14 g (14.4 mmoles) of Boc_2O are dissolved in 13 ml of water/ethanol mixture (1/1). After dissolution of the reagents, 1.58 ml of N-methylmorpholine (14.4 mmoles) are added to the reaction medium. After stirring for 2 hours at room temperature, the mixture is diluted in 50 ml of water. The aqueous phase is saturated with KH_2PO_4, then extracted with petroleum ether (2 x 30 ml) and diethyl ether (3 x 30 ml). The organic phases are collected, then dried on sodium sulfate and finally concentrated under reduced pressure. The obtained product 2 is a

yellow oil (2.66 mg, 12 mmoles, yield: 93,7%) used without any further purification in the following synthesis. The NMR analysis of the product 2 is the following : NMR ^1H ($CDCl_3$, ref TMS, 323 K) δ : 4.19 (q, 2H, J=7 Hz), 4.11 (s, 2H), 1.45 (m, 9H), 1.26 (t, 3H, J=7.16 Hz).

- *Synthesis of ethyl N,N'-tri(Boc)hydrazino-acetate 3*

Compound 2 (3.26 g, 14.9 mmoles) is dissolved in 3 ml of CH_2Cl_2, in the presence of 4.36 ml of Et_3N (31.29 mmoles) at 0°C. Furthermore, 6.83 g (31.29 mmoles) of Boc_2O are dissolved in 5 ml of CH_2Cl_2 in the presence of 546 mg (4.47 mmoles) of DMAP at 0°C. After complete dissolution of the reagents, the compound 2/Et_3N mixture is added, dropwise, to the Boc_2O/DMAP mixture. As soon as the addition is completed, the temperature of the reaction medium is progressively reduced to room temperature. After stirring for 2 hours, the medium is diluted with 10 ml of CH_2Cl_2. The organic phase is washed with a solution saturated with KH_2PO_4, dried on sodium sulfate then distilled under reduced pressure. The yellow-orange residual oil is purified by chromatography on silica (40-60 microns) with a CH_2Cl_2/AcOEt mixture (97:3). The obtained product 3 is a yellow oil (3.0 g, 7.2 mmoles, yield: 48 %). The NMR analysis thereof is the following : NMR ^1H (DMF_{d7}, ref TMS, 330 K) δ : 4.18 (s, 2H), 4.16 (q, 2H, J=7 Hz), 1.46 (m, 27H), 1.22 (t, 3H, J-7 Hz).

The analysis of product 3 by mass spectrometry is the following :

MALDI-TOF $[M+H]^+$ calculated : 419.5, found : 441.4 $[M+Na]^+$, 457.4 $[M+K]^+$.

- *Synthesis of N,N'-tri(Boc)hydrazinoacetic acid 4*

Compound 3 (3.0 g, 7.2 mmoles) is subjected to a treatment with a mixture consisting of 10,8 ml of 1M soda and 10 ml of ethanol, at room temperature. The

mixture is stirred for 2h30 at room temperature. The reaction medium is further diluted with 20 ml of water, extracted into a basic medium with 2 x 20 ml of ether, then acidified by addition of 1N hydrochloric acid. Then the aqueous phase is extracted with dichloromethane (2 x 20 ml) and further with diethyl ether (2 x 20 ml). The organic phases are collected, dried on Na_2SO_4, filtered and concentrated under reduced pressure. The residual mixture is recrystallized in a mixture of diethyl ether/heptane (2/3). The obtained product 4 is a white solid (1.7 g, 4.4 mmoles, yield: 61%). The NMR analysis thereof is the following: NMR ^1H (DMF_{d7}, ref TMS, 330 K) δ : 4.20 (s, 2H), 1.47 (brs, 27H); NMR ^{13}C (DMF_{d7}) 169.7 (C=O), 150.9 (C=O), 85.7 (quaternary C), 51.8 (CH_2), 27.9(CH_3).

2) Synthesis of N,N'-di(Boc)hydrazinoacetic acid 4'

• *Synthesis of ethyl N-Boc hydrazinoacetate 2*

14.9 g (96.4 mmoles) of commercial ethyl hydrazinoacetate 1 and 26.1 g (119.5 mmoles) of Boc_2O are dissolved in 70 ml of a water/ethanol mixture (1/1). After dissolution of the reagents, the reaction medium is cooled down to 0°C. 13.2 ml of N-methylmorpholine (119.5 mmoles) are added dropwise. After stirring for 15 minutes at 0°C, then for 2h at room temperature, the mixture is diluted in 100 ml of water. The aqueous phase is saturated with KH_2PO_4 then extracted into diethyl ether (2 x 70 ml) and petroleum ether (2 x 70 ml). The organic phases are collected, the dried on anhydrous sodium sulfate and finally concentrated under reduced pressure. The obtained product 2 is a yellow oil (19.8 g. 91.1 mmoles, yield: 94.5%). This is dried on phosphorus pentoxide (P_2O_5) overnight. The thus obtained product 2 is used without further purification in the following synthesis. The NMR analysis of the product 2 is the following: NMR ^1H ($CDCl_3$, ref TMS, 323 K) δ : 4.19 (q, 2H, J=7.18 Hz),

4.11 (s, 2H), 1.45 (m, 9H), 1.26 (t, 3H, J=7.16 Hz). NMR ^{13}C ($CDCl_3$ ref TMS, 323 K) δ : 175.51 and 174.38 (C=O), 161.76 and 160.83 (C=O), 85.89 and 85.23 (quaternary C), 65.57 and 65.46 (OCH_2CH_3), 57.41 (CH_2CO), 32.87, 32.78 and 32.63 ($(CH_3)_3C$), 18.70 (CH_2CH_3).

Elementary analysis of the product 2 (total formula): $C_9H_{18}N_2O_4$) : C 49.53, H 8.31, N 12.84, O 29.32 (calculated), C 49.78, H 8.36, N 12.33, O 29.27 (found).

• *Synthesis of ethyl N,N'-tri(Boc)hydrazino-acetate 3*

Compound 2 (19.86 g, 91.1 mmoles) is dissolved in 16 ml of CH_2Cl_2 under inert atmosphere, in the presence of 38.5 ml of Et_3N (276 mmoles) at 0°C. Furthermore, 60.2 g (276 mmoles) of Boc_2O are dissolved in 20 ml of CH_2Cl_2 in the presence of 3.4 g, (27.6 mmoles) of DMAP at 0°C. After complete dissolution of the reagents, the compound 2/Et_3N mixture is added dropwise to the Boc_2O/DMAP mixture under inert atmosphere, at 0°C. As soon as the addition is completed, the temperature of the reaction medium is progressively reduced to room temperature. After stirring for 2h, the medium is diluted with 50 ml of CH_2Cl_2. The organic phase is washed with a solution saturated with KH_2PO_4 (3 x 75 ml), dried on sodium sulfate then concentrated under reduced pressure. The residual yellow-orange oil is purified by filtration on silica (40-60 microns, 160 g) with a CH_2Cl_2/AcOEt mixture (97:3). The residual yellow oil is dried overnight in the presence of P_2O_5. 36.9 g (88.3 mmoles ; yield: 96.9%) of product 3 are obtained.

The NMR analysis of product 3 is the following : NMR ^1H $CDCl_3$, ref TMS, 300 K) δ : 4.16 (s, 2H), 3.71 (q, 2H, J=7 Hz), 1.46 (m, 27H), 1.23 (t, 3H, J= 7Hz). NMR ^{13}C ($CDCl_3$ ref TMS, 300 K) δ : 167.74 (C=O), 150.48 and 150.23 (C=O), 83.68, 82.46 and 82.00 (quaternary C), 60.98 and 58.40 (OCH_2CH_3), 53.51 and 51.57 (CH_2CO),

28.09 ($(CH_3)_3C$)), 18.42 and 14.20 (CH_2CH_3).

Elementary analysis of product 3 (total formula): $C_{19}H_{34}N_2O_8$) : C 54.53, H 8.19, N 6.69 (calculated), C 54.81, H 8.25, N 6.71 (found).

The analysis of product 3 by mass spectrometry is the following: MALDI-TOF $[M+H]^+$ calculated : 419.5 found : 441.4 $[M+Na]^+$, 457.4 $[M+K]^+$.

- *Synthesis of N,N'-di(Boc)hydrazinoacetic acid 4'*

The compound 3 (36.9 g, 88.3 mmoles) dissolved in 135 ml of ethanol is treated with 135 ml molar soda at 0°C. After stirring for 30 minutes at 0°C, the temperature of the reaction medium is progressively reduced to room temperature. The mixture is stirred for 3h30 at room temperature. Afterwards, the reaction medium is dissolved with 110 ml of water and extracted into diethyl ether (2 x 80 ml). The aqueous phase is acidified by addition of 1N hydrochloric acid until pH 2. The reaction is exothermic. The aqueous phase is then extracted with dichloromethane (2 x 80 ml), diethyl ether (2 x 80 ml). The organic phases are collected, washed with a solution saturated with KH_2PO_3, dried on anhydrous Na_2SO_4, filtered and finally concentrated under reduced pressure. The residual mixture is maintained overnight at 4°C, then cold recrystallized in a diethyl ether/heptane mixture (90 ml/120 ml). The obtained product 4' is a white solid (17.9 g, 61.6 mmoles, yield 69.8 %). The NMR analysis thereof is the following : NMR ^1H (DMF_{d7}, ref. TMS, 330 K) δ: 4,20 (s, 2H), 1,47 (brs, 27H) ; NMR ^{13}C (DMF_{d7}) 169,7 (C=O), 150,9 (C=O), 85,7 (quaternary C), 51,8 (CH_2), 27,9 (CH_3).

EXAMPLE 2: Synthesis and purification of hydrazinopeptide 6 (Figure 2)

- Synthesis of hydrazinopeptide 6

Peptide 5 is synthesized on a Wang resin (0.73

mmole/g, Applied Biosystems, Foster City, USA), according to the Fmoc/*tert*-butyl strategy as described, for example, by FIELDS *et al.*, in *Int. J. Pept. Protein*, 1990, 35, 161, and a HBTU/HOBt activation (see SCHNÖLZER *et al.*, in *Int. J. Pept. Protein Res.*, 1992, 40, 180), with use of a peptide synthesizer Applied Biosystem 431A (Foster City, USA). Protections of the side chain are: His(Trt), Glu(O^tBu), Arg(Pmc), Lys(Boc). At the end of the synthesis, the Fmoc group of the arginine α-NH_2 function is displaced in the presence of piperidine 20% in DMF. Afterwards, N,N'-tri(Boc) hydrazinoacetic acid 4 (1.2eq) is manually introduced using the BOP activation in *situ* (BOP 1.2 eq, DIEA 3.6 eq in DMF for 20 min), as described for example by GAIRI *et al.*, in Tetrahedron Letters, 1990, 50, 7363. The N,N'-di(Boc)hydrazinoacetic acid could also be used as a variant. The peptidyl-resin is washed successively with DMF, dichloromethane, and ether. Afterwards, the resin is dried under reduced pressure for 30 min.

The cleavage of the peptide-resin bond as well as the deprotection of the side chains are carried out in the presence of a TFA/H_2O/anisole mixture (1 g of dried resin/9.5 ml of TFA/0.25 ml of anisole/0.25 ml of H_2O) under stirring for 2h at room temperature. Peptide 6 is precipitated in a Et_2O/heptane mixture (1/1 previously cooled down to 0°C (200 ml). The precipitate is centrifuged, then dissolved in a H_2O/AcOH mixture (5/1), deep-frozen and freeze-dried.

- Purification of hydrazinopeptide 6

Hydrazinopeptide 6 was purified by HPLC on a C18 hyperprep column using a linear gradient from 0 % to 50 % of a TFA/water/isopropanol mixture (ratio water/isopropanol 2/3, the mixture containing 0,05 % of TFA) in a 0.05 % TFA/water mixture. Such an eluent advantageously enables to avoid any decomposition of the peptide. The purified compound is freeze-dried and stored at -20°C.

The purity of the purified compound is controlled by analytical HPLC on a C18 Vydac column using the same eluent system as previously. The identity of peptide 6 was controlled by ES-MS analysis with a Micromass Quatro spectrometer ($[M+H]^+$ calculated 1432.5, found 1432.7).

• Characterization of the peptidyl-resin 5

Prior to introduction of the N,N'-tri(Boc)hydrazinoacetic acid 4, the amino acid composition of peptide 5 was controlled by total hydrolysis carried out on peptidyl-resin in the presence of a 6N hydrochloric acid/propionic acid mixture (1/1) and a few drops of phenol, at 140°C, for 3h. This hydrolysis is followed by identification on an amino acid analyser Beckman, Model 7300.

EXAMPLE 3 : Synthesis of lipopeptides 11, 12, 13, 14, 16 and 18 (Figure 3).

1) Synthesis of compounds 7, 8, 9, 10, 15 and 17

• *Synthesis of compounds 7, 8, 9, 15 and 17.*

Where R (Figure 3) is the fatty chain of an oleic acid, 10 mg (35.4 µmoles) of oleic acid, 4.08 mg (35.4 µmoles) of N-hydroxysuccinimide and 4.3 µl (27.2 µmoles) of diisopropylcarbodiimide are dissolved in a THF/dichloromethane mixture (175 µl/175 µl). After a night at 0°C, the medium is concentrated under reduced pressure. The residual oil (compound 8) is taken up in 6.8 ml of 2-methyl-propane-2-ol.

The same process is used for activating the palmitic, stearic, linoleic and cis-9,10-epoxystearic acids, i.e. for obtaining these acids as succinimidyl esters (obtaining compounds 7, 9, 17 and 15).

• *Synthesis of compound 10.*

500 mg (1.13 mmoles) of cholesteryl chloroformate and 140.9 mg (1.22 mmoles) of N-hydroxysuccinimid are dissolved in 2 ml of dichloromethane at room temperature. 170 µL (1.22 mmoles) of triethylamin are

added to the reaction medium. The reaction is exothermic and a whitish precipitate is formed. After stirring for 45 min at room temperature, the medium is diluted with 50 ml of dichloromethane and washed with a solution saturated with KH_2PO_4. The organic phase is dried on sodium sulfate, filtered, then concentrated under reduced pressure. The obtained compound 10 is a white solid (451.6 mg, 0.85 mmoles, yield: 76%). It is a cholesteryl carbonate activated with N-hydroxysuccinimide.

2) Synthesis of lipopeptide 11

- Protocol

6 mg (3 (μmoles) of hydrazinopeptide 6, the synthesis thereof was described in Example 2, are dissolved in 900 μl of a 0.25 mM phosphate/citrate buffer pH = 5.2 (160,2 μl of a 0.2 M Na_2HPO_4 solution and 139.8 μl 0.1M of citric acid topped up to 1.2 ml with water). The pH of hydrazinopeptide 6 in solution is readjusted, if necessary, with the 0,2M Na_2HPO_4 solution. 1.48 mg (3.6 μmoles) of succinimidyl palmitoate 7 are dissolved in 900 μl of 2-methyl-propane-2-ol. Afterwards, both solutions are mixed and stirred at room temperature for 72 h.

The use of a mixed medium buffer/2-methyl-propane-2-ol allows both to control the pH of the reaction medium and to ensure the favourable solubility of hydrazinopeptide 6, of fatty acid 7 and of final lipopeptide 11. In addition, the introduction of the lipophilic part on the peptide is carried out under mild conditions, thus enabling the introduction of fatty acids sensitive to strong acids.

The progress of the reaction is monitored by HPLC on a C3 Zorbax colum (0 to 100% of solvent B with 0.05% TFA/80 % acetonitril/20% water for 30 min, then 5 min at 100 % of solvent B, 1 ml/min, detection at 215 nm). After 72 h, the monitoring by HPLC shows the end of the reaction. Then the reaction medium is diluted with 5 ml

of a water/acetic acid mixture (80/20) and purified on a C3 Zorbax column using the previous eluent system. After deep-freezing and freeze-drying, the lipopeptide 11 is obtained in a 61 % yield (3.89 mg, 1.83 μmoles). Only 6% of lipopeptide diacyl are obtained (coupling of the palmityle group not only to the hydrazine hydrazine group of peptide 6, but also to the amine function located on the lysine residue side chain of said peptide).

• Characterization of lipopeptide 11.

The purified compound is subjected to an ES-MS (Micromass Quatro 11 Electrospray Mass Spectrometer) analysis. $[M+H]^+$ calculated : 1672.1, found : 1671.6.

The NMR TOCSY analysis confirms the structure of the product 11. The sample is prepared by dissolving the lipopeptide 11 in 500 μl of a CD_3CO_2H/H_2O mixture (80/20). The concentration of final peptide 11 is 5 mM. The chemical displacements are given in relation to the sodium salt of the 3-(trimethylsilyl)[2,2,3,3-$_{d4}$]-propionic acide, used as internal standard. The NMR spectra are obtained on a Bruker DRX 300 a 300 K.

3) Synthesis of lipopeptides 12, 13, 14, 16 and 18

The process is similar to that described in 2) for the lipopeptide 11 synthesis by reacting hydrazinopeptide 6 with the compounds 8, 9, 10, 15 and 17.

Only the purification of lipopeptide 16 varies. The purification thereof by HPLC is performed at a pH 7.0 on C3 Zorbax column using the following eluent: from 100 % of solvent A (50 mM phosphate buffer, pH 7.0) to 100 % of solvent B (50 mM phosphate buffer, pH 7.0, comprising 50 % of isopropanol) for 100 minutes, at a rate of 3 ml/minute and at 50°C, the detection is carried out at 215 nm. The thus obtained compound 16 is then desalted using the following conditions: polystyrene-divinylbenzene column, gradient from 100 %

of solvent A (water containing 0,05 % of triethylamine) to 100 % of solvent B (mixture water/acetonitril 20/80 containing 0,05 % of triethylamine) for 10 minutes, at a rate of 4 ml/minute and at 50°C, the detection is carried out at 215 nm.

The characterization of lipopeptides 12, 13, 14, 16 and 18 by ES-MS and the obtained yields of different lipopeptides are resumed in the following (Table I) :

Table I

lipopeptide	lipophilic group	$[M+H]^+$ calculated	$[M+H]^+$ found	yield
12	oleyl	1697.2	1697.8	53%
13	stearyl	1699.2	1699.5	65%
14	cholesteryl	1845.6	1845.7	56%
16	cis-9,10-epoxy-stearyl	1713.2	1713.5	53%
18	linoleyl	1695.2	1695.5	51%

Only 6, 7 and 8 % of biacylated lipopeptides are obtained respectively by the synthesis of lipopeptides 12, 13 and 14.

EXAMPLE 4 : Synthesis of lipopeptide 13 by catalytical hydrogenation of lipopeptide 12 (Figure 4).

500 µg of palladium 10 % on carbon suspended in 600 µl of a 20 % solution of concentrated acetic acid in water are added to 5 mg (2.3 µmoles) of compound 12, obtained as described in the previous example, dissolved in 300 µl of the same solution. After stirring for 4 hours at room temperature under hydrogen atmosphere, 1.64 mg of 10 % palladium on carbon suspended in 100 µl of pure acetic acid are added to the reaction medium. After 20 h, the conversion is complete and the medium is filtered on celite and washed with a solution of 20 % acetic acid in water (3x3 ml), then with methanol (3x3 ml). The filtrate is concentrated under reduced pressure, deep-frozen and freeze-dried. The thus obtained compound is purified by

HPLC on a C3 Zorbax column using a linear gradient from 0 % to 55 % of a water/acetonitril/TFA mixture (1/4 of water/acetonitril, with 0,05 % of TFA) in a 0.05 % TFA/water mixture (water containing 0,05 % TFA). The purified compound (2.55 mg, 1.2 mmoles, yield: 52%) is freeze-dried and stored at -20°C.

The purity of the purified compound is controlled by analytical HPLC on a C3 Zorbax column using the same eluent system as previously. The compound is identified by ES-MS : $[M+H]^+$ calculated : 1699.2, found : 1699.6.

EXAMPLE 5 : Synthesis of lipopeptide 21 (Figure 5).

1) Synthesis of hydrazinopeptide 19.

Hydrazinopeptide 19 was synthesized on 0,25 mmole (357.1 mg) of Rink Amide aminomethyl-polystyrene resin containing 1 % of divinylbenzene (0,70 mmole/g, 100-200 Mesh, Senn Chemicals AG) using the *Fmoc/tert*-butyl strategy as described for example in FIELDS *et al.*, *Int. J. Pept. Protein*, 1990, 35, 161, and a HBTU/HOBt activation (SCHNÖLZER *et al.*, *Int. J. Pept. Protein Res.*, 1992, 40,180), using a peptide synthesizer Applied Biosystem 431A (Foster City, USA). The Fmoc protecting groups are removed with a piperidine solution. At the end of the synthesis, the terminal N-lysine α-NH_2 function Fmoc protecting group is removed using a 20 % piperidine solution in DMF.

The thus deprotected α-NH_2 function is modified using the solid phase electrophilic N-amination process developed by C. KLINGUER *et al.* (Tetrahedron Letters, 1996, 37, 40, 7259-7262). The obtained hydrazinopeptide is deprotected and cleaved from the resin with 10 ml of a TFA solution (94 % TFA, 2,5 % H_2O, 2,5 % thioanisole, 1 % triisopropylsilane) for 1h30 under stirring. Afterwards the compound is precipitated in 100 ml of a Et_2O/pentane 1/1 solution. After precipitation and removal of the supernatant, the pellet is dissolved in a 10 % acetic acid, deep-frozen and freeze-dried.

The identity of hydrazinopeptide 19 is controlled by PDMS-TOF on a mass spectrometer with Plasma Bio-ion Desorption 20. $[M+H]^+$ calculated : 895.5, found : 895.9.

The purification of hydrazinopeptide 19 is carried ou on a preparative C3 Zorbax column (30°C, detection at 235 nm, buffer A= H_2O 100%/TFA 0,05%, buffer B - isopropyl alcohol 40 %/H_2O 60 %/TFA 0,05 %, flow rate 2 ml/minute, from 0 to 70% of B for 70 minutes). After deep-freezing and freeze-drying, hydrazinopeptide 19 is obtained in a 56 % yield. The purity of the product after freeze-drying is controlled by RP-HPLC under same conditions as previously described.

2) Synthesis of lipopeptide 21.

5.06 mg of hydrazinopeptide 19 are dissolved in 791 µl citrate-phosphate buffer pH 5.11. 1.1 eq. (4,12 µmole) of succinimidyl palmitate 20 (Su being a succinimidyl group) dissolved in 791 µl of ^{t}BuOH are then added. The reaction is monitored by RP-HPLC on a C3 Zorbax column. 48 h later, the reaction medium is purified on a preparative C3 Zorbax column (30°C, detection at 215 nm, buffer A = H_2O 100 %/TFA 0,05 %, buffer B = acetonitril 80 %/H_2O 20 %/TFA 0,05 %, flow rate 3 ml/minute, from 0 to 70 % of B for 70 minutes). The lipopeptide 21 is obtained in a yield of 60 %.

EXAMPLE 6 : Synthesis of lipopeptides 23 and 24 (Figure 6).

1) Synthesis of hydrazinopeptide 22.

- Synthesis protocol

Peptide 22 synthesized on a Fmoc-PAL-PEG-PS resin (0,16 mmole/g, Perseptive) following the Fmoc/*tert*-butyl strategy and a HBTU/HOBt activation (see Example 2) using a peptide synthesizer Pioneer-Perseptive. The protections of the amino acid side chain are the following : His(Trt), Asn (Trt), Glu(O^{t}Bu), Arg(Pbf), Lys(Boc), Ser(^{t}Bu). At the end of synthesis, the Fmoc

group of the alanine α-NH_2 function is removed in the presence of 20 % piperidine in DMF. The N,N'-tri(Boc)hydrazinoacetic acid (1,2 eq.) is the manually introduced using the BOP activation *in situ* (BOP : 1,2 eq., DIEA : 3,6 eq. in DMF for 20 minutes). The peptidyl-resin is washed successively with DMF, dichloromethane, and ether. The resin is dried under reduced pressure for 30 minutes. The cleavage of the peptide-resin bond and the deprotection of side chains as well are carried out in the presence of a TFA/phenol/ethanedithiol/thioanisole/H_2O mixture (1 g dried resin/10 ml TFA/0,25 ethanedithiol/0,25 ml H_2O/0,25 ml thioanisole/0,75 g phenol) under stirring for 3h30 h at room temperature. The peptide is precipitated in 200 ml of a Et_2O/heptane (1/1) mixture previously cooled down to 0°C. The precipitate is subjected to a centrifugation, then dissolved in a H_2O/AcOH (5/1) mixture, deep-frozen and freeze-dried. 263 mg of raw peptide are obtained from 0,072 mmole of resin.

- Purification hydrazinopeptide 22.

Hydrazinopeptide 22 was purified by HPLC on a C3 Zorbax column using a linear gradient from from 0 % to 50 % for 70 minutes of a 0,05 % TFA/water/isopropanol mixture (2/3) in a mixture of 0,05 % TFA/water. The purified compound (43 mg) is freeze-dried and stored at -20°C. The hydrazinopeptide 22 analysis by ES-MS is the following : $[M+H]^+$ calculated : 4645.5, found : 4645.7.

2) Synthesis of lipopeptides 23 and 24.

Compounds 7 and 10 are prepared as described in Example 3. Lipopeptides 23 and 24 are obtained from compounds 7 and 10 respectively and from hydrazinopeptide 22, according to the process previously described for the lipopeptide 11 synthesis. They were obtained in a yield of 40 % after purification.

ES-MS (Micromass Quatro II Electrospray Mass

Spectrometer) analysis yields the following results :

lipopeptide 23 : $[M+H]^+$ calculated : 4883.5, found : 4883.7 ;

lipopeptide 24 : $[M+H]^+$ calculated : 5058.5, found : 5059.0.

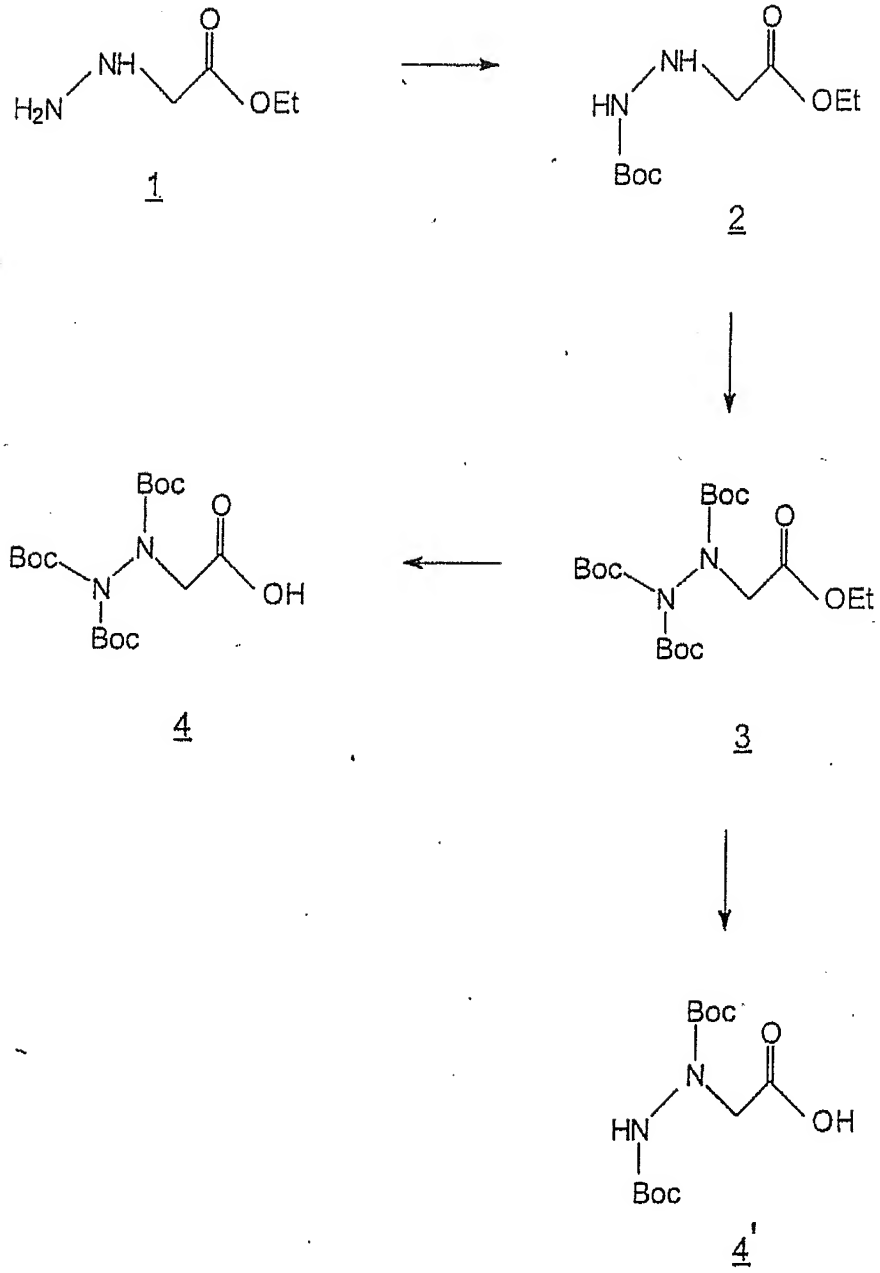
As it emerges from the foregoing description, the invention is not limited to performing, embodiments and applications described in details ; on the contrary, it encompasses all variants appreciated by those skilled in the art, without departing from the frame, the scope and the extent of the present invention.

CLAIMS

1) The use of N,N'-tri(Boc)hydrazinoacetic acid or of N,N'-di(Boc)hydrazinoacetic acid for functionalizing a peptide with an α-hydrazinoacetic group, either at the N-terminal end of said peptide, or at the end of a lysine or an ornithine side chain possibly present at any point of the peptide sequence.

SUMMARY

The present invention is related to the use of N,N'-tri(Boc)hydrazinoacetic acid or of N,N'-di(Boc)hydrazinoacetic acid for the functionalization of a peptide with an α-hydrazinoacetic group.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1
NH
HN
Boc
O
OEt
2
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Boc
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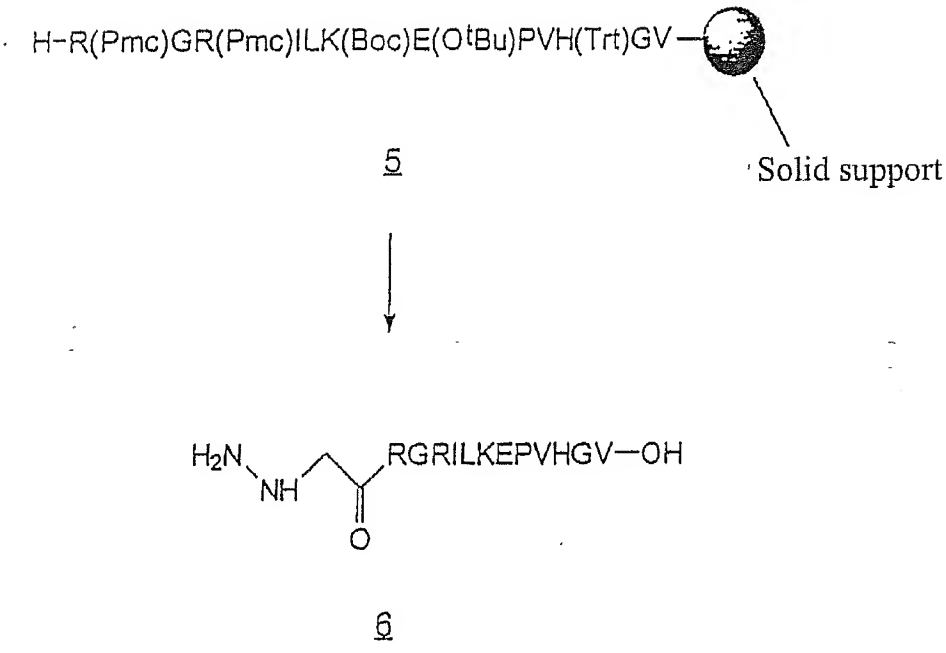
OH
4'

FIGURE 1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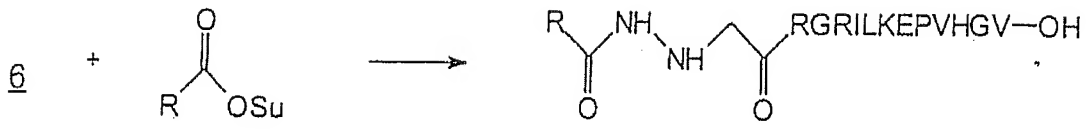
NH
O
RGRILKEPVHGV—OH
6

FIGURE 2



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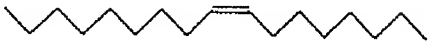

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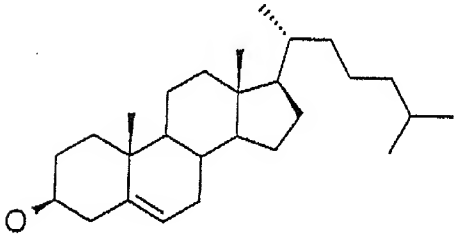

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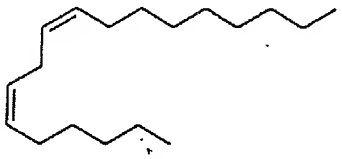

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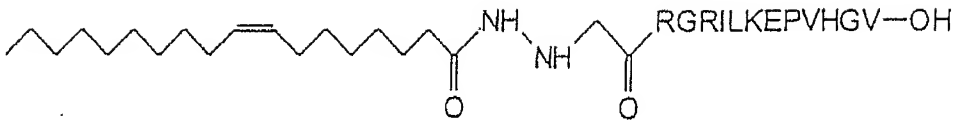
FIGURE 3



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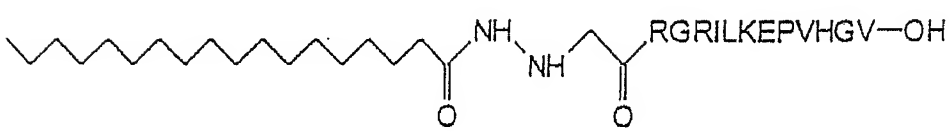

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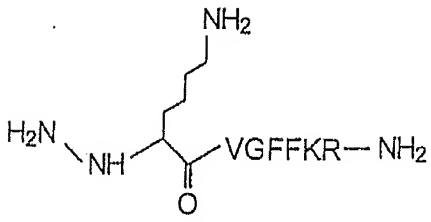
FIGURE 4



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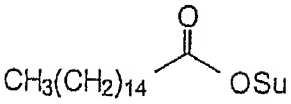

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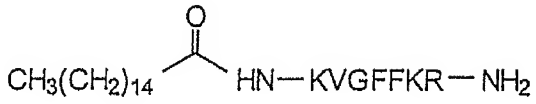

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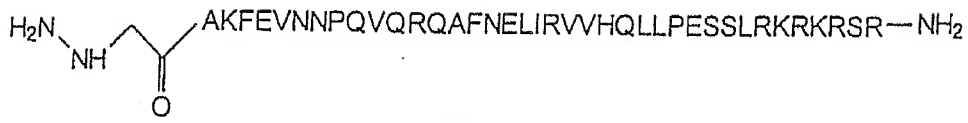
FIGURE 5



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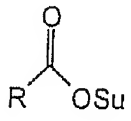

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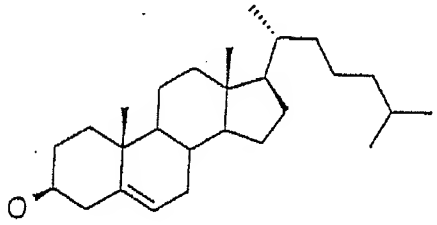
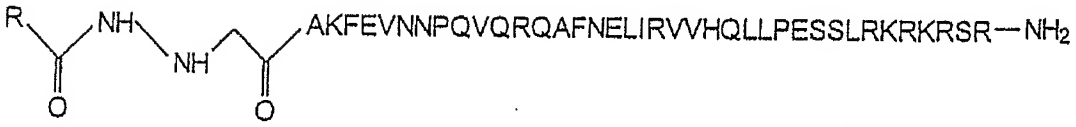
R =  10



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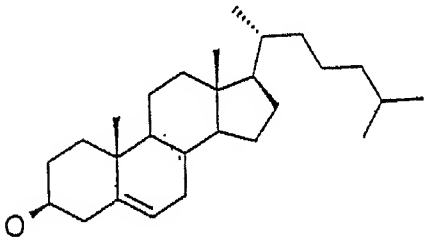
R =  24

FIGURE 6